A DISSERTATION REPORT ON

SCREENING AND CHARACTERIZATION OF MICROBIAL CONSORTIA FOR PRODUCTION OF CHITINASE ENZYME.

Submitted to the

DEPARTMENT OF BIOSCIENCES

INTEGRAL UNIVERSITY

Submitted By

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M. Sc Microbiology (Semester IV)

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प्रमाण पत्र

श्री अर्पित आचार्या, पुत्र श्री रमेश आचार्या एम.एससी. माइक्रोबायोलॉजी इंटीग्रल यूनिवर्सिटी, लखनऊ ने दिनांक 18.02.2022 से दिनांक 22.06.2022 तक **"Screening and characterization of microbes for chitinase production using agri waste as substrate"** शीर्षक में डा. शुचि श्रीवास्तव, प्रिंसिपल साइंटिस्ट के पर्यवेक्षण (Supervision) में सफलतापूर्वक प्रशिक्षण प्राप्त किया।

CERTIFICATE

Mr. Arpit Acharya, S/o Shri Ramesh Acharya, student of M.Sc. Microbiology, Integral University, Lucknow has successfully completed the training from 18.02.2022 to 22.06.2022 on the project entitled, **"Screening and characterization of microbes for chitinase production using agri waste as substrate"** under the supervision of Dr. Suchi Srivastava, Principal Scientist.

for. 22/6/22
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DECLARATION

I, **ARPIT ACHARYA**, hereby declare that the project report entitled **"SCREENING AND CHARACTERIZATION OF MICROBES FOR THE PRODUCTION OF CHITINASE ENZYME ."**, submitted to **Department of Biosciences, Integral university Lucknow** in partial fulfillment of the requirements of the Degree of Master of Science in Microbiology, is an independent work carried out by the undersigned during a period of four months, under the guidance and supervision of **Dr. Suchi Srivastava, Principal Scientist, CSIR-National Botanical Research Institute**, **Lucknow** and to the best of knowledge and belief this report has not forms on the basis of award of any Degree/Diploma/Associate/Fellowship or other similar title to any candidate of any university.

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CERTIFICATE BY INTERNAL ADVISOR

This is to certify that **Mr. Arpit Acharya** a student of **M.Sc. Microbiology** (2nd year/4th semester), Integral University Lucknow, has completed his four month dissertation work entitled successfully.

He has completed this work from ***CSIR-NBRI*** (National Botanical Research Institute) LUCKNOW under the guidance of **Dr. Suchi Srivastava**. The dissertation was a compulsory part of his M.Sc. Microbiology degree.

I wish his good luck and bright future.

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TO WHOM IT MAY CONCERNS

This is to certify that **Arpit Acharya** a student of M.Sc. Microbiology (IV Semester) Integral University has completed his four months dissertation works entitled **"SCREENING AND CHARACTERIZATION OF MICROBIAL CONSORTIA FOR PRODUCTION OF CHITINASE ENZYME**" successfully. He has completed this work from 18-02-2022 to 22-06-2022 under the guidance of **Dr. Suchi Srivastava** (Principle Scientist) **Division of Microbial Technology CSIR NBRI Lucknow**. The dissertation was a compulsory part for the award of his M.Sc. Degree in Microbiology

I wish him bright and a great future.

(Dr Snober S. Mir)

Associate Prof & Head

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List of instrument and kit used

S. NO.	Name of the instruments/ kits	Company
1.	Freezer	Sanyo
2.	Horizontal Autoclave	Equitron
3.	Hot Air Oven With Temperature Control	Universal
4.	Innova4320 Incubator Shaker	New Brunswick Scientific
5.	Microwave	L.G.
6.	MR 33i Centrifuge	Thermo Scientific
7.	Weighing Balance	Mettler Toledo
8.	Micropipette	Gilson
9.	Vortex	Genei
10.	Laminar Air Flow	-
11.	UV- Trans Illuminator	Thermo Scientific
12.	UV-Torch3	Genei
13.	Thermal Cycler	Techno
14.	Gel Doc	Thermo Scientific

Chapter -1

INTRODUCTION

I. Background:

Agriculture is an important sector of Indian economy as it contributes about 17% of the total GDP. India produces 450-500 million tons of crop residues annually. As per estimates, approximately 19-20 million tons of paddy straw, 12 million tons of corncob and about 20 million tons of wheat straw are produced annually. Farmers generate several metric tons of agricultural wastes per year and start burning tons of hay in a very short time to prepare their land for the next season resulting in a pall of smoke known as "Black cloud", a mass of polluted air across the country. Black cloud, comprise of a mix of volatile organic compounds, carcinogenic substances, sulphur oxides, nitrogen oxides and carbon monoxide, is responsible for about 42% of autumnal air pollution **(**El-Ghonemy DH, 2015**).** About 85-90 percent of this paddy straw and 75 percent of corncobs is burnt in the field are also burnt and also used in the industrial field

Paddy straw, the only organic material available in significant quantities contain 40% of the nitrogen, 30-35% of the phosphorus, 80-85 % of the potassium and 40-50 % of the sulphur of the total nutrients taken up by plants' vegetative parts. Paddy straw has a plenty of potential uses still unexplored; the gathered rice straw is being used in various different industrial processes as a renewable resource. **Corn**, the second largest agricultural commodity followed by rice. In the past few years, corn has gained considerable importance as one of the economic sources for the production of cellulose. However, thousands of tons of corncob remain unused as agricultural wastes. Corncob has highlighted for the dramatically increased in the population of agricultural waste. **Bagasse** Sugar-cane bagasse is a fibrous waste-product of the sugar refining industry, along with ethanol vapor. This waste product (Sugar-cane Bagasse ash) is already causing serious environmental pollution, which calls for urgent ways of handling the waste (R. Srinivasan, 2010).

Microbes are known to produce several hydrolases of industrial importance. These microbial enzymes have enormous focus due to their widespread application in various sectors of pulp, paper, and agriculture. A variety of microorganisms, including bacteria, yeast and filamentous fungi have been reported to produce cellulases and xylanases, in which the most potent producers are *Aspergillus oryzae, Trichoderma sp*., *Bacillus sp*. and *Streptomyces sp*. (S.I. Mussatto, 2010) In order to improve the quality and quantity of the

production, selection of efficient strain and their improvement through mutational approaches are the important step. The present proposal is based on the use of a combination of native micro-flora with high efficiency of degradation property. Combination of these microorganisms having faster degradation property would be potent thereby possessing the ability of producing better enzyme activity (A Khootama, 2018). Therefore, this report mainly focuses on the idea to standardize the conditions for better production of chitinase enzyme from microbial sources using different agri-waste as a substrate.

These crop residues are massive value to the farmers and can play important role to revive the economy of a country (G. Allesina, 2018), therefore, there is a need to devise a suitable technology for agri-waste management to convince the farmers not to burn it. Paddy straw, corn cob and bagasse have a plenty of potential uses that are still unexplored; the gathered agri-waste can be used in various different industrial processes as a renewable resource.

1.1. OBJECTIVES:

- Screening and characterisation of microbial strains for chitinase enzyme production.
- *In -vitro* compatibility test of the selected bacterial and fungal strains.
- Selection of microbial consortia for better enzyme production using agri- waste as a substrate.
- Quantitative estimation of microbial chitinase enzyme using rice straw, corncob and bagasse as source of carbon.

Literature Review

Chapter – 2

Agriculture has a major share in the overall economy of India. Being the dominant area of Indian economic system it employs more than 50-55% of population in India. According to Indian economic survey 2018, it contributes to around 17-18% country's GDP In distinct agro-ecological area of India, a wide range of crops are cultivated across the vast majority of land (K Sukhadia, 2019) After crop harvesting, the left over plant material including leaves, stalks and roots was known as crop residue and India generates around 500 Metric ton of crop residue annually (GOI, 2016).According to the report from Devi et al., different crops produce distinct amount of crop residues such as 110 Metric ton by wheat, 122 Metric ton by rice, 71 Metric ton by maize, 26 Metric ton by millets, 141 Metric ton by sugarcane, 8 Metric ton by fiber crops (jutemesta, cotton) and 28 Metric ton by pulses (Devi et al., 2017).

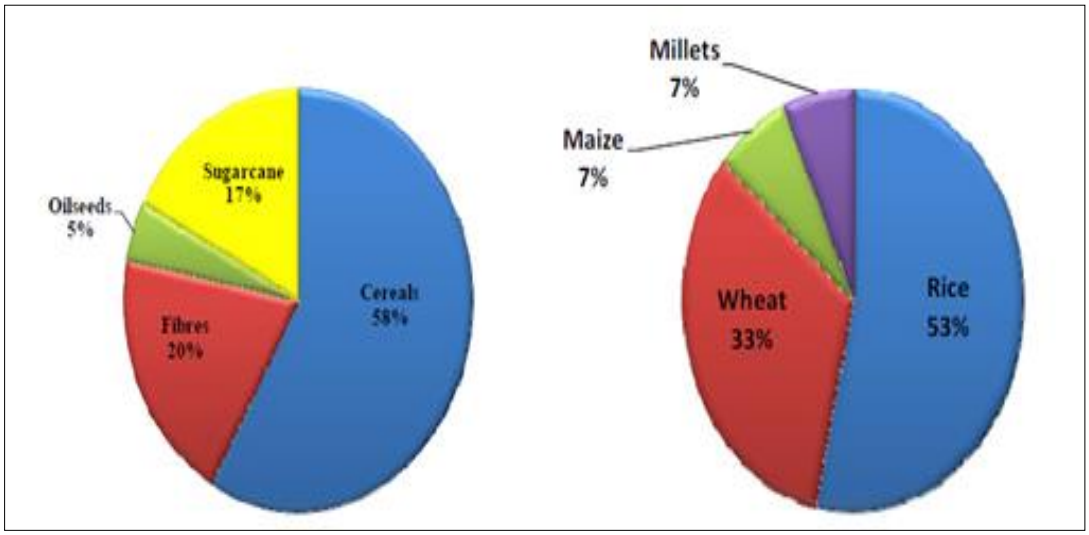

Figure 1 (a) Contribution of crops in residue generation. (b) Contribution of different cereal crops in residue generation (Source: Jain et al., 2014)

India reportedly produces around 110 Metric ton of rice, 170 Metric ton of rice straw every year (Pandey, 2019). When cereal crops are harvested, it is estimated that half of the process ends with agricultural waste or crop residue as straw (V Smil , 1999) Agro industries are also generating huge amount of solid and liquid waste. Discharge of these wastes into the environment without proper treatment causes serious environmental problems (Y.C. Ho, 2012). Hence it needs to be treated or reused. The potential for food processing waste as raw material or for conversion into useful and higher value added

products is high. These wastes can also be used as food or feed after biological treatment. About 70% of the agro waste is turned into waste during the canning operation (A Bhatnagar, 2015) these wastes contain high content of carbohydrate that can be utilized for the production of organic acids, alcohols and enzymes. The utilization of industrial and agricultural waste produced by industrial processes has been the focus of waste reduction research for economic, environmental, and technical reasons (M Duque-Acevedo, 2020).

The largest share among all agricultural by-products is acquired by paddy straw. Rice (paddy) straw is rice by- product produced after harvesting paddy (Verma, et, al. 1992) Each kg of milled rice produced results in roughly 0.7–1.4 kg of rice straw depending on varieties, cutting-height of the stubbles, and moisture content during harvest (Singh et. al. 2015). Rice straw is separated from the grains after the plants are threshed either manually, using stationary threshers or, more recently, by using combine harvesters. The by-product comprises of only organic material available in significant quantities *viz.* 40% of the nitrogen, 30-35% of the phosphorus, 80-85 % of the potassium and 40-50 % of the sulphur of the total nutrients taken up by plants' vegetative parts (AL Meena,2021) Different enzyme production techniques using biotechnological methods involve high production cost, whereas rice straw being a renewable and economic source (N Sarkar et, al..2012).

Figure 2: Harvesting grain and straw using combined harvester

The corncob is an agricultural waste generated in huge quantities during corn processing. Corn, the second largest agricultural commodity compared to rice and highlighted for the dramatically increased agricultural waste (E. Aprianti, 2015) In the past few years, corn has gained considerable importance as one of the economic sources that are recycled and reused as bio products for the production of cellulose and other hydrolytic enzymes. However, thousands of tons of corncob remain unused as agricultural wastes and a large amount remains unused and burned in the fields (W. T. Tsai, 1997). In 2020, corn production for

India was 30,250 thousand tons. Corn production of India increased from 5,101 thousand tons in 1971 to 30,250 thousand tons in 2020 growing at an average annual rate of 4.67%.

Figure 3: Corn cob as a agricultural waste and farmer's practice for management of corn cob

Sugar-cane bagasse is a third most abundant fibrous waste-product from the sugar refining industry, along with ethanol vapor. This waste product (Sugar-cane Bagasse ash) is causes serious environmental pollution, which calls for urgent ways of handling the waste (R Srinivasan, 2010). The natural, bio-degradable features and chemical constituents of the sugarcane bagasse (SCB) have been attracting attention as a highly potential and versatile ingredient in composite materials. Eco-friendly and low cost considerations have set the momentum for material science researchers to identify green materials that give low pollutant indexes (Y.R. Loh,2013) Various components of SCB are shown to possess the ability of being applied as raw material for manufacturing of composite materials at multiple levels of properties and performances. Studies on the impacts, performances and applications of SCB in its original condition; transformed forms; treated with appropriate chemicals and/or processes; in combination with materials of distinct properties and manipulation of manufacturing methodologies have been duly considered. (T.C. Sarker, 2017).

Sugarcane bagasse is a highly promising source of biomass in the Indian context for the establishment of a bio-based economy due to their highly nutrient value, the nutrient content of sugarcane bagasse consists of 52% cellulose, 35.5% hemicellulose, 1.5% total nitrogen, 29% carbon and C/N ration is 23% in total sugarcane (R. Sun, 2010). Due to presence of large amount of Carbon components, bagasse can be used to produce a number of value-added products such as organic acids, ethanol, bio-catalysts and specialty chemicals such as vanillin (S. Soru - 2019). Utilization of sugarcane bagasse, therefore, poses a sustainability challenge with food, energy and water nexus. Cellulose and hemicellulose from bagasse are available for subsequent processes like chemical or

enzymatic hydrolysis leading to improved fermentation procedure for production of biological components (KJ Dussán, 2014).

Sugarcane bagasse is a vegetal biomass that has much potential for use because of its structural elements: cellulose, hemicellulose and lignin (GJ de Moraes Rocha, 2015). In order to use it as a raw material to produce new compounds, sugarcane bagasse needs to undergo a pre-treatment process, leading to the removal of lignin from the vegetable fibers for example, in the production of 2° generation ethanol. The methodology of delignification of biomass using ScCO2 is a clean process that aims at the non-formation of intermediate residues (KA Khalid, 2017).

Fermentation Technology

Fermentation is the technique of biological conversion of complex substrates into simple compounds by various microorganisms such as bacteria and fungi. In the course of this metabolic breakdown, they also release several additional compounds apart from the usual products of fermentation (R. Subramaniyam, 2012). These additional compounds are called secondary metabolites. Secondary metabolites range from several antibiotics to peptides, enzymes and growth factors. The development of techniques such as Solid State Fermentation (SSF) and Submerged Fermentation (SmF) has led to industrial-level production of bioactive compounds.

Submerged fermentation: Submerged Fermentation (SmF)/Liquid Fermentation (LF) SmF utilizes free-flowing liquid substrates, such as molasses and broths (Daverey A. Pakshirajan, K. (2009). It is a method of manufacturing biomolecules in which enzymes and other reactive compounds are submerged in a liquid (TB Dey, 2016). Submerged fermentation involves the growth of the microorganism as a suspension in a liquid medium in which various nutrients are either dissolved or suspended as particulate solids in many commercial media (Nakahara, T., 2010). It results in the production of industrial enzymes, antibiotics or other products. The process involves taking a specific microorganism such as fungi and placing it in a small closed flask containing the rich nutrient broth. (MM Al-Ghanem, 2018). A high volume of oxygen is also required for the process. The production of enzymes then occurs when the microorganisms interact with the nutrients on the broth resulting in them being broken down. The bioactive compounds are secreted into the fermentation broth (E. Dimidi, 2019).

Submerged fermentation technology has the advantages of short period, low cost and high yield. Purification of products is easier, in liquid culture the control of the fermentation is

simpler and consequently significant reductions in fermentation times can be achieved. In the same way, the use of submerged culture can benefit the production of many secondary metabolites and decrease the production costs by reducing the labour involved in solid-state methods.

Solid state fermentation: Solid-state fermentation (SSF) is defined as the fermentation involving solids in absence (or near absence) of free water; however, substrate must possess enough moisture to support growth and metabolism of micro-organism. Solid-state fermentation has emerged as a potential technology for the production of microbial products such as feed, fuel, food, industrial chemicals and pharmaceutical products (Ashok Pandey, 2003) Utilisation of agro-industrial residues as substrates in SSF processes provides an alternative avenue and value-addition to these otherwise under- or non-utilized residues. SSF stimulates the growth of micro-organisms in nature on moist solids and has been credited to be responsible for the beginning of fermentation technique in ancient time (Siti Maftukhah, 2019). There are several important aspects, which should be considered in general for the development of any bioprocess in SSF. These include selection of suitable micro-organism and substrate, optimization of process parameters and isolation and purification of the product. Going by theoretical classification based on water activity, only fungi and yeast were termed as suitable micro-organisms for SSF. It was thought that due to high water activity requirement, bacterial cultures might not be suitable for SSF. However, experience has shown that bacterial cultures can be well managed and manipulated for SSF processes (Mahanta et. al, 2008)

The advantages of SSF over Submerged Fermentation (SmF) are indicated as the economic feasibilities of adopting SSF technology in the commercial production of industrial enzymes such as amylases, cellulases, xylanase, proteases, lipases, etc., organic acids such as citric acid and lactic acid, and secondary metabolites such as gibberellic acid, and antibiotics such as penicillin, cyclosporin, cephamycin and tetracyclines are highlighted. The relevance of applying SSF technology in the production of mycotoxins, biofuels, and biocontrol agents is discussed, and the need for adopting SSF technology in bioremediation and biotransformation of agro-products and residues is emphasized.

It has been generally claimed that product yields are mostly higher in SSF in comparison to SmF. However, so far there is not any established scale or method to compare product yields in SSF and SmF in true terms. The exact reasoning for higher product titres in SSF is not well known currently. The logical reasoning given is that in SSF microbial cultures are closer to their natural habitat and probably hence their activity is increased.

Enzyme production using Agri-waste as a Substrate

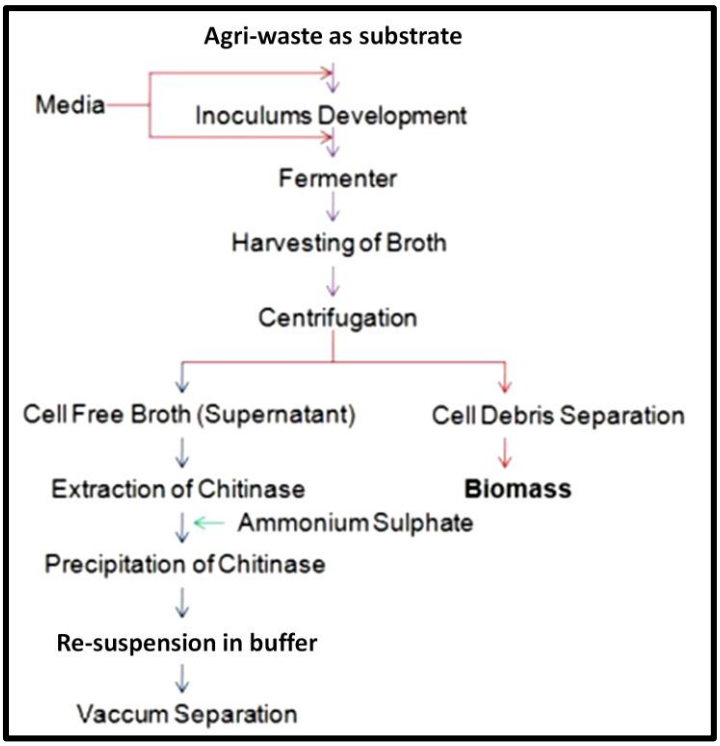

Figure 4: Enzyme production using fermentation

Agriculture waste are abundant source of lignocellulose, and other saccharifying sugars available worldwide that, in past, were treated as waste in many countries. With the increasing expansion of agro industrial activities, large quantities of ligno-cellulosic residues are generated annually. Ligno-cellulosic biomass is mainly found at the cellular wall and, is composed of cellulose, hemicelluloses and lignin, along with organic acids, salts and minerals. Therefore, such residues are desirable substrate for growth of filamentous fungi which produce cellulolytic, hemicellulolytic and ligninolytic enzymes by Solid state fermentation (SSF), as their hyphae can profusely grow on the surface of particles and can easily penetrate through the inter particle spaces for colonization (Geoffrey M. Gadd, 2006) Filamentous fungi are the most significant producer of enzymes involve in the degradation of ligno-cellulosic material (Mathew et al.. 2008)

The development of biotechnological methods based on enzyme production could provide a renewable resource to recycle rice straw for several industrial application. High cost of production is perhaps the major constraint in commercialization of new sources of enzymes. Therefore, using an inexpensive substrate such paddy straw, corncob, bagasse, etc. which regarded as agri-waste by majority of countries, use of high yielding microbial strains, mutagenesis, optimal fermentation condition and efficient enzyme recovery procedure can reduce the cost and economize the process of enzyme production (Ghonemy

et al., 2014a). My report mainly focuses on the production of Chitinase enzyme using agriculture waste as a substrate using microbial consortia.

Chitinase: Many bacteria and fungi produce extracellular chitinolytic enzymes, known as chitinases (E.C. 3.2.1.14), able to convert chitin into compounds that can be of industrial interest, including N-acetyl-D-glucosamine.

Chitin, a homo-polymer of N-acetyl-D-glucosamine (Glc-NAC) residues linked by β-1,4 bonds, and its derivatives, hold great economic value because of their versatile biological activities and agrochemical applications (Muzzarelli et al.,2012). Chitinase has also received attention due to its use as a bio-control agent. These enzymes have been used in biological research for the generation of fungal protoplasts to degrade the fungal cell wall, and are also used in human health care such as making ophthalmic preparations with chitinases. There is an increasing interest in the use of chitinases for the control of plant diseases caused by various phyto-pathogenic fungi, insects, nematodes, and the production of different chitin oligomers (Huang et al. 2005). The main drawback is the high cost of chitinase production, and this enhances the necessity to search for high yielding enzyme-productive strains and inexpensive cultivation media (Muzzarelli et al. 2012).; Several species of fungi such as *Trichoderma harzianum* and *Aspergillus sp.* (Rattanakit et al., 2002), and with bacteria and actinomycetes such as *Bacillus subtilis B. cereus*, *B. licheniformis* and *Streptomyces* have shown a chitinase producing ability. The use of microorganisms to process agri-waste offers a waste management solution and commercial rewards.

Applications of Chitinases: Chitinases have applications in many different areas some of them are mentioned below:

Chitinases are attaining prominence in the field of biotechnology applied in waste management, pest control in agriculture, and human health care which have been recapitulated in Figure 4.

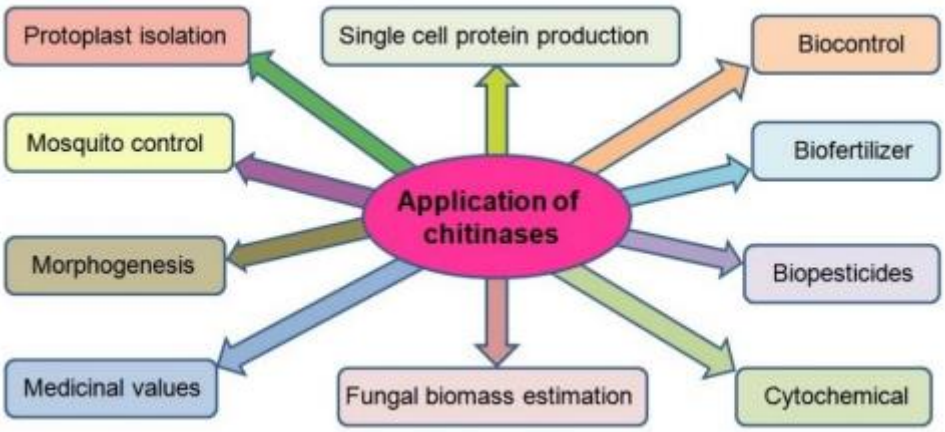

Figure 5: Application of chitinase.

Waste Management: Microbial chitinases can be used to convert chitinous biomass, that is, chitinous waste of marine organisms into simpler useful depolymerized components, hence reducing water pollution (Abhishek Singh Rathore, 2015) Chito-oligomers obtained by action of chitinases have a wide range of biotechnological applications in biochemical, food, and various chemical industries. Chitinase can also be used in conversion of chitinous waste into biofertilizers (Raj Singh, 2020). Another approach to utilize the chitinous waste effectively is production of single cell protein (SCP). In this approach chitinase degraded chitinous waste is used as carbon or nutritional source for production of biomass. Chitinase producing bacteria and yeast can be used in aqua cultures for SCP production (Rinkoo D. Gupta 2015)

Biocontrol Agents: Chitinases are present in plants along-side various pathogenesis related proteins as a part of plant defence mechanism (Saboki Ebrahim, 2011) Chitinase can also be directly used as biopesticides against various fungi and insects that can be an alternative to chemical pesticides. Other than being used directly as a biocontrol agent, chitinase can act as a target for biopesticide as chitin has a major role in insect metamorphosis as well as in gut of insects. In agriculture, the inhibitory properties of chitinases are being harnessed for the biological control of pests and fungal diseases of plants. The control of fungal phyto-pathogens such as *Trichoderma reesei* , *Colletotrichum gleosporoides*, *Rhizoctonia solani* , *Fusarium oxysporum*, and *Fusarium graminarium* has been reported.

Medical Application: Chitinase is used as antifungal agent in combination with antifungal drugs in therapy for various fungal infections. Chitinase is also being suggested to be used for detection of invasive fungal infection in humans. Chito-oligosaccharides also have an enormous pharmaceutical potential to be used in human medicines because of its antitumor activity (shown by chito-hexose and chito-heptose), wound healing property. Acetyl

glucosamine, which is a monomeric unit of chitin polymer, is also reported to be anti-inflammatory agent (R. A. A. Muzzarelli, 1997)

Miscellaneous Applications: Chitinases have been exploited to isolate fungal protoplasts that are used as experimental means to study the synthesis of cell wall, synthesis and secretion of enzymes, and strain improvement for biotechnological applications (Aneesa Fasim, 2020). The level of chitinases can also be used for the indirect determination of fungal biomass present in the soil. An enzyme of food industry purpose tannase is produced by *Aspergillus niger* but tannase binds to its cell wall reducing the yield. Chitinase is used for fungal cell wall degradation that releases tannase from cell wall and increases the yield (Hartmut Kuhn, 2015)

Chitin derivatives	Process of synthesis	Applications
Chitosan	Deacetylation	Anticancer, antibacterial agent, enzyme immobilization,
Alkyl chitin	Deacetylation of chitosan	Antimicrobial agents
N and O sulfated chitin	Sulfation	Anticoagulant agent, heparin
Dibutyryl chitin	Reaction of the chitin with butyric anhydride	Intermediates for further chemical modifications
Carboxymethyl chitin (CMCH)	Carboxymethylation	Excipients for oral drug delivery
Chito-oligosaccharides (COS)	Acid hydrolysis and oxidative, reductive depolymerization	Nutraceutical additive
Chitin nanofibers (CNF)	Loosening of tightly bonded fibrils bundles by removal of minerals, proteins, pigments, and lipids with the treatment of HCl, NaOH, and ethanol	Tissue engineering wound dressing, cosmetic, skin health, stem cell, anticancer therapy, drug delivery, obesity treatment, anti-inflammatory
Chitin nano-whiskers (CNW)	Treatment of chitin with 3N HCl at 100°C or 3M H_2SO_4 solution	Nanotechnology and nanocomposite material for drug/gene delivery or nanoscaffolds in tissue engineering

Figure 6: Chitin derivatives and its applications

Bio-ethanol production from Agriculture Wastes:-

Due to abundancy of agri-waste and its high carbon content, they have another major industrial usage in production of bio-ethanol. It has high cellulose and hemicelluloses content that can be readily hydrolyzed into fermentable sugars. Bioethanol produced from agriculture waste can be used as transportation fuel. But there occur several challenges and limitations in the process of converting Agriculture waste to ethanol. The presence of high ash and silica content in Agriculture waste makes it an inferior feedstock for ethanol production. One of the major challenges in developing technology for bioethanol production from Agriculture waste is selection of an appropriate pre-treatment technique. (Binod et al., 2010) reviewed the current available technologies for bioethanol production from rice straw. The choice of pre-treatment methods plays an important role to increase

the efficiency of enzymatic saccharification thereby making the whole process economically viable.

Degradation of the cellulosic complex such as paddy straw, corn cob and bagasse to simple sugars can be brought about with the help of microorganism like brown rot and soft rot fungi (Akhtar, 2015) Biological pre- treatment renders the degradation of lignin and hemicelluloses and white rot fungi (WeiWang, 2013) seem to be most effective microorganism. Several microorganisms are used for the fermentation of saccharified (residual) biomass (Katarzyna Robak, 2018) But for commercially viable production, the process requires the ideal microorganism or the potent microbial consortia which have broad substrate utilisation efficiency, high ethanol yield and productivity, should have the proficiency to withstand high temperature, should be tolerant to inhibitors present in hydrolysate and have cellulolytic activity. Some microorganisms usually employed in the fermentation include *Saccharomyces cerevisiae, Escherichia coli, Zymomonas mobilis, Pichia stipitis, Candida brassicae, Mucor indicus*, etc (Sarkar et al., 2012).

Chapter - 3

MATERIALS & METHODS

3.1 List of reagent used:

Serial No.	Name of reagent	Preparation
1.	Saline (0.85%)	0.85gm NaCl in 100 ml DW and autoclaved
2.	Colloidal Chitin	Using Hydrochloric acid
3.	Lignin	Lignin sulphonic acid
4.	Potassium ferricyanide	5.25gm sodium carbonate in 100ml DW then 0.1 gm potassium ferricyanide was mixed in sodium carbonate
5.	Laminarin solution	0.5% laminarin prepared in 0.5M phosphate buffer: pH 7(1M K_2HPO_4 and KH_2PO_4)
6.	Dinitrosalicylic acid (DNS)	Solution A- sodium potassium tartarate in DW Solution B- DNS in 2N NaOH Mix sol. A and sol. B

Media composition:

1. NUTRIENT BROTH COMPOSITION:

Components	Amount (gm/l)	Preparation
Beef extract	1	Suspend the following components in 1 litre of DW. Heat this mixture while stirring to fully dissolve all components. Sterilised by autoclaving at 121°C for 15 min.
Peptone	5	
Yeast extract	2	
Sodium chloride	5	

2. CZAPEX DOX BROTH COMPOSITION:

Components	Amounts (gm /L)	Preparation
Sucrose	30.00	Suspend the following in 1 L on D.W. Heat if necessary to dissolve the medium
Sodium nitrate	3.00	
Dipotassium phosphate	1.00	

Magnesium sulphate	0.500	completely. Mix well and despense into flasks as desired Sterilized by autoclaving at15 lbs pressure (121°C) for 15Minutes.
Ferrous sulphate	0.010	
Potassium chloride	0.500	

3. NUTRIENT AGAR MEDIA COMPOSITION:

Components	**Amounts (gm/l)**	**Preparation**
Peptone	5	Suspend the following components in 1 litre of DW. Heat this mixture while stirring to fully dissolve all components. Autoclave the dissolved mixture at 121°C for 15 min.
Beef extract	3	
Agar	15	
Sodium chloride	5	

4. Potato Dextrose Agar

Components	**Amounts (gm/l)**	**Preparation**
Potatoes , infusion form	200	Suspend the following components in 1 litre of DW. Heat this mixture while stirring to fully dissolve all components. Autoclave the dissolved mixture at 121°C for 15 min
Dextrose	20	
Agar	15	

5. ROSE BENGAL AGAR COMPOSITION:

Components	**Amount (gm/l)**	**Preparation**
Papaic digest of soyabean meal	5.00	Suspense 31.55 gram in 1L DW. Heat this mixture while stirring to fully dissolve all components. Autoclave the dissolved mixture at 121°C for 15 min.
Dextrose	10.00	
Monopotassium phosphate	1.00	
Magnesium sulphate	0.500	
Rose bengal	0.050	
Agar	15.000	

6. CHITINOLYTIC MEDIA COMPOSITION:

Components	Amount (gm/l)	Preparation
Magnesium sulphate heptahydrate.	0.3	The components were mixed and resulting substrate had a yellow bright color, and retained enough bromocresol purple even after pH is adjusted to 4.7 and autoclaved for 15 mins at 121°C temperature and 15 lb/inch^2 pressure.
Di-ammonium sulphate	0.3	
Citric acid (anhydrous)	0.91	
Potassium di-hydrogen orthophosphate	2.0	
Tween-80	200 µl	
Colloidal Chitin	4.5	
Bromocresol purple	0.15	
Agar-agar type-1(2%)	20	

3.1 Procurement of materials:

Isolation of microbes: Bacterial and Fungus strains used were isolated from the compost samples, degraded wood and litters present in CSIR-NBRI Garden and other different sites. For bacteria, the samples were subjected to serial dilution upto 10^{-4} and 100µl of this dilution was spread on NA plates and for fungi, the samples were subjected to serial dilution upto 10^{-2} and 100µl spread on RBA plates. Both plates were incubated at 28° C for 48 hour and morphological distinct colonies of bacteria and fungus were isolated on separate NA and PDA plates respectively.

3.2 Screening and characterization of bacterial and fungal strains

The bacterial and fungal isolates that were obtained from isolation were to be characterized for qualitative and quantitative screening for chitinase production.

3.2.1 Qualitative assay for determination of chitinase activity:

Chitinolytic media plates containing colloidal chitin as a substrate and Bromocresol purple as a pH indicator dye were poured on sterile petri plates and bacterial strains were stabbed while fungal mycelium was placed on the plates. These plates were incubated at 28ºC for 72 hrs. After incubation the chitinase activity was identified by measuring the purple colored zone in vicinity of fungus.

3.2.2 Quantitative assay for determination of chitinase activity:

Selected strains were characterized for enzyme production using Czapek Dox Broth media supplemented with 0.5% colloidal chitin. Bacterial and fungal cultures were inoculated simultaneously in the conical flask. The flasks were incubated at 28˚C for 48 hours on shaker. Sampling was done at 3rd, 5th and 7th Day of inoculation for estimation of chitinase enzyme production.

After sampling, the cultures were transferred into eppendorfs and centrifuged at 6000rpm for 10 min at 4˚C. 100µl of the supernatant (enzyme extract), 100µl 2% chitin, 100µl sodium acetate buffer (150mM: pH 6.1) and 200µl distilled water were mixed in borosilicate test tubes. The 500µl prepared reaction mixture was incubated at 37˚C for 1 hour. 500µl of 0.5M NaOH added in order to stop the reaction followed by addition of 1ml of potassium ferricyanide. The tubes were incubated for 15 min in boiling water (100˚C) and measure the absorbance at 420nm by setting the blank with distilled water. One unit of chitinase activity can be defined as the amount of enzyme required to release 1µmol of N-acetyl glucosamine residue per ml of extract per minute.

3.3 Fermentation Process for Chitinase Production

Chitinase production using agri- waste has been carried out using two different fermentation processes *viz.* Submerged fermentation and solid-state fermentation.

For submerged fermentation was set up using 5% Rice straw and 5% corncob as a substrate and inoculated with the highest chitinase producers and one control treatment was used which solely includes Czapek Dox Broth media with 0.5% colloidal chitin and for Solid State Fermentation 25 gram of mentioned substrates' were added with same microbial strains and moisture is maintained accordingly. Sampling for the extract was done at 3rd, 5th, 7th, 10th, 15th and 20th day.

5gm of treated extract was suspended in 20 ml of sodium phosphate citrate buffer for enzyme extraction. The extract obtained was further used for quantitative estimation.

3.4 *In-vitro* compatibility test for efficient consortia:

The use of suitable microbial combination (compatible fungal and bacterial strain) to produce cell wall degrading enzyme mainly chitinase potentially offers the opportunity to increase enzyme production and to acquire more robust and diverse enzyme cocktails in a

single step by combined action of microorganisms With this thought the compatibility between selected microbes was studied to develop efficient microbial consortia.

Highest chitinase producing strains of the fungus and bacteria were inoculated on NA+PDA plates and kept for incubation at 28^0C overnight. 6mm circular discs of fungus were inoculated over PDA and placed in the centre of the plate. The bacterial strains were streaked at the edges of plate after 48 hour incubation of fungus. The plates were incubated at 28˚C till the maximum growth attained. Bacterial strains possessing in-compatibility will show the zone of inhibition against fungi or flattening of growth of fungus.

3.5 Up-scaling process of production of Chitinase enzyme:-

Up-scaling process is the large scale production of chitinase enzyme. Among SSF and SMF, Solid state fermentation proves more reliable and efficient process for production of chitinase enzyme. Three different types of substrates are used *viz.* corn cob, rice straw and bagasse for enzyme production. To set up a fermentation process 100gm of each substrate is used and moisture is maintained. The flasks are autoclaved and 48 hour grown culture of fungi was inoculated in the same flasks. It was kept for incubation at 28º C and after 48 hour the bacterial culture was inoculated in the same flask. Sampling was done at 3^{rd}, 5^{th}, 7^{th} and 10^{th} day. The sampled extract was used for estimation of chitinase production by above stated protocol.

3.6 Estimation of degradative enzymes

In order to synthesize chitinase enzyme, the microbe should utilize organic source provided to it. Utilization of organic biomass can be accomplished by its breakdown into simpler forms and this requires certain enzymes *viz.* cellulase for the breakdown of cellulose/ hemi-cellulose and laccase for lignin degradation. The extract used for chitinase estimation in up-scaling process was further used for the estimation of both cellulase and laccase enzymes.

Laccase enzyme was quantified using ABTS prepared in sodium acetate buffer mixed with extract and incubated at 28º C for 30 min and absorbance recorded at 420nm. The cellulase activity was quantified using Carboxymethyl cellulose prepared in sodium phosphate buffer mixed with extract and incubated at 37º C for 60 min. The reaction was terminated using DNS and boiled for 15 min. Absorbance for the same was recorded at 540nm.

3.7 Extraction of Crude Enzyme

Crude enzyme was extracted from fermented substrate using sodium citrate phosphate buffer in ratio 1:4. The solid substrate with buffer solution was kept on shaker for 2 hour. The extract was filtered using whattman filter paper and 100 ml of this extract was saturated with 70% ammonium Sulphate and kept at 4º C for 2 hour. Extract was centrifuged at 10000 rpm for 15 min and the obtained pellet was re-suspended in 0.1M Sodium acetate buffer. Aliquots were prepared for suspended crude enzyme and preserved at -80º C.

3.8 Evaluation of crude enzyme for antifungal activity

The antifungal nature of crude enzyme was evaluated using poison food assay which involves supplementation of crude enzyme in the fungal growth media. For testing the effective concentration of crude enzyme activity, different concentrations of enzyme was used *viz.* 25µl, 50µl, 100µl, 200µl and 500µl. The liquid media supplemented with crude enzyme was inoculated with a phyto-pathogenic fungus. In this evaluation, *Rhizoctonia solani* that causes leaf blight disease of rice was used.

3.9 Molecular characterization of selected strains

3.9.1 Extraction of genomic DNA from bacterial and fungal cultures:

Total genomic DNA was extracted using the protocol given by Maniatis et al. (1982) which was later modified by Sadowsky et al. (1987).

1 ml of 24 hours old bacterial and fungal culture was centrifuged at 10,000 rpm for 10 minutes at 4°C and pellet was suspended in TAE buffer.

- The suspension was centrifuged again at 10,000 rpm and responded in 500 µl of 0.5 mg/ml lysozyme in TAE. After 10 minutes of incubation at RT
- Then it was suspended in 500 µl of 2.3% w/v SDS and 0.2 mg/ml of Proteinase k.
- The mixture was incubated at 55°C for 2 hours.
- 5 µl of RNase was added to the suspension and incubated for 30 minutes at 37° C.
- Equal volumes of phenol and chloroform : iso-amyl alcohol (24:1) were added to the suspension, centrifuged at 10,000 rpm,
- The upper layer of the solution thus obtained was extracted twice with chloroform and iso-amyl alcohol (24:1) .
- The extract was precipitated using 1/10 volume of 3 M sodium acetate and 2.5 volume of ethanol and centrifuged at 10,000 rpm for 30 min.

- The pellet was washed with 70% ethanol, air dried and finally re-suspended in 50µl TE buffer.

The elute contains pure Genomic DNA. For short term storage 2-8ºC is recommended and for long term storage -20ºC is recommended.

3.9.2 Gel Electrophoresis of genomic DNA

Gel electrophoresis of the genomic DNA was carried out in a submarine horizontal agarose slab gel apparatus as described by Sambrook et al. (1989). Agrose 0.8% was suspended in 1X TAE buffer, boiled until clear solution was obtained. Molten agarose was allowed to cool and ethidium bromide (0.5µg/ml) was added to it. It was poured in an electrophoresis tray and allowed to cast at room temp. Slots were made by fixing slot former over the tray, before pouring the molten agarose. The comb was removed after the agarose become solidified and the gel was transferred to an electrophoresis tank containing 1x TAE buffer. The DNA samples mixed with tracking dye (0.001 M EDTA pH 8.0, glycerol 50%, Bromophenol blue 0.4% and xylene cyanal FF 0.4% were loaded in slots. After completion of the electrophoresis at 90 v/cm, the DNA bands were visualized in UV Gel-Doc Trans illuminator.

Chapter - 4

RESULTS AND DISCUSSION

4.1 Isolation of bacterial and fungal strains:

Approximately 200 bacterial and 70 fungal strains were isolated from different samples (degraded woods, litters, agri-compost, etc.) and were characterized for their complex-carbon degrading ability to produce chitinase enzyme. The initial screening of microbial strains involves chitinolytic activity in solid media and then the quantification of the enzyme produced by individual strain using liquid growth media under optimum conditions.

4.2 Screening and characterization of microbial strains:

There were 54 bacterial strains and 61 fungal strains showing positive activity. The activity was identified by the formation of purple coloured zone. Coloured zone and intensity were taken as the criteria to determine the chitinase activity after 2-3 days of incubation.

The principle behind the formation of colored zone is that the media is supplemented with a pH indicator dye bromocresol purple which converts the yellow color of the media (acidic pH 4.7) into purple due to increase in pH towards alkalinity. The pH increases because of the utilisation of chitin by the microbes and its breakdown into product N-acetyl glucosamine (chitin being a homo-polysaccharide of N- acetyl glucosamine), which is basic in nature and thus cause for corresponding shift in pH towards alkalinity (Agrawal, T. and Kotasthane, A.S., 2012).

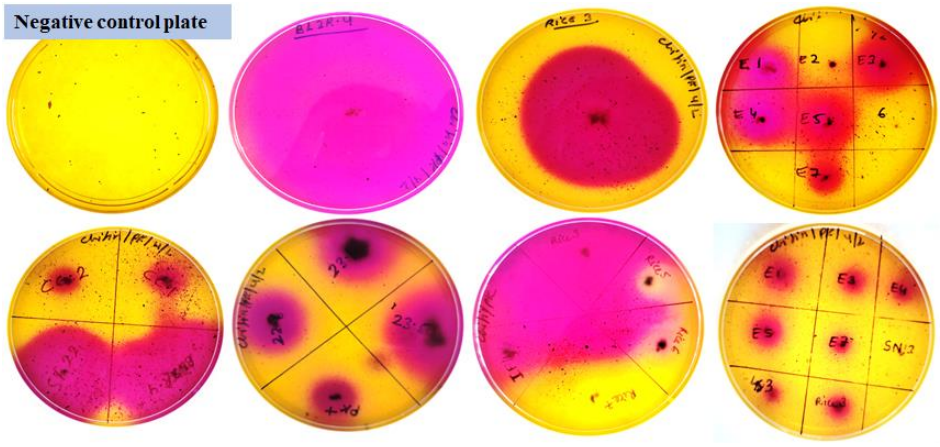

Figure 7: Qualitative Assay for chitinase enzyme production

Table 1: Table showing chitinolytic activity of different isolated strains

STRAINS	CHITINOLYTIC ACTIVITY	ZONE DIAMETER	STRAINS	CHITINOLYTIC ACTIVITY	ZONE DIAMETER
MG 11.1	-	0	MG 17.4	-	0
MG 11.2	-	0	MG 17.5	-	0
MG 11.3	-	0	MG 17.6	-	0
MG 11.4	-	0	MG 17.7	-	0
MG 11.5	-	0	MG 17.8	-	0
MG 11.6	-	0	MG 17.9	-	0
MG 11.7	-	0	MG 18.1	-	0
MG 12.1	-	0	MG 18.2	-	0
MG 12.2	+	1.2	MG 18.3	-	0
MG 12.3	-	0	MG 18.4	-	0
MG 12.4	-	0	MG 18.5	-	0
MG 12.5	-	0	MG 18.6	-	0
MG 12.6	-	0	MG 18.7	-	0
MG 13.1	-	0	MG 18.8	-	0
MG 13.2	+	1.4	MG 21.4	+	0.7
MG 13.3	-	0	MG 21.6	+	0.6
MG 13.4	-	0	MG 21.7	+	1.4
MG 13.5	-	0	MG 21.8	+	0.8
MG 13.6	-	0	MG 22.3	-	0
MG 13.7	-	0	MG 22.5	-	0
MG 13.8	-	0	MG 22.6	+	1.4
MG 14.1	+	1.2	MG 23.1	+	1.4
MG 14.2	-	0	MG 23.2	-	0.7
MG 15.1	-	0	MG 23.3	+	0.9
MG 15.2	-	0	MG 23.4	+	1.8
MG 15.3	-	0	MG 23.5	+	0.7
MG15.4	-	0	MG 23.6	+	0
MG 15.5	-	0	MG 23.7	-	0.4
MG 15.6	-	0	MG 23.8	+	0.4
MG 16.1	-	0	MG 24.1	+	1.3
MG 16.2	-	0	MG 24.2	+	0
MG 16.3	-	0	MG 24.3	-	1.9
MG 16.4	-	0	MG 24.5	+	0.9
MG 16.5	-	0	MG 24.6	+	1.1
MG 16.6	-	0	MG 24.7	-	0
MG 16.7	+	0.5	MG 24.8	-	0
MG 16.8	-	0	MG 24.9	+	0.5
MG 17.1	-	0	PK +	+	1.8
MG 17.2	-	0	BL2R.3	+	1.3
MG 17.3	-	0	M2D.1	-	0

Table 2: Table showing chitinolytic activity of different isolated bacterial strains

STRAINS	CHITINOLYTIC ACTIVITY	ZONE DIAMETER	STRAINS	CHITINOLYTIC ACTIVITY	ZONE DIAMETER	STRAINS	CHITINOLYTIC ACTIVITY	ZONE DIAMETER
3.13 (P)	+	0.7	**C.1.1(c)**	+	1	**BLR.7**	+	0.8
12.7 (P)	-	0	**12.8 (C)**	-	0	**BLR.8**	+	1.2
7.7 (P)	-	0	**C.1.1(c)**	+	1	**BLR.9**	+	0.6
3.16(P)	+	0.5	**12.8 (C)**	-	0	**BLR.10**	++	1.6
4.1 (P)	+	1	**CD.1**	-	0	**BLR.11**	+	0.7
8.9 (P)	+	1.8	**CD.2**	-	0	**BLR.12**	-	0
8.1 (P)	+	0.6	**CD.3**	++	1.6	**BL.1**	-	0
4.6 (P)	-	0	**CD.4**	-	0	**BL.2**	+	0.8
7.15 (P)	+	0.5	**CD.5**	+	1	**BL.3**	-	0
11.2 (P)	+	0.5	**CD.6**	-	0	**BL.4**	-	0
9.4 (P)	-	0	**CD.7**	-	0	**BL.5**	-	0
w2.14 (L)	-	0	**CD.8**	-	0	**BL.6**	-	0
8.8 (L)	+	1.6	**CD.9**	-	0	**BL.7**	-	0
7.3 (P)	+	0.5	**CD.10**	-	0	**BL.8**	-	0
4Su.10	+	1.6	**CD.11**	-	0	**BL.9**	+	0.5
3.8 (P)	-	0	**CD.12**	+	0.7	**BL.10**	+	0.4
3.13 (c)	+	0.5	**CD.13**	+	0.4	**BLS.1**	-	0
4.7(P)	+	1.5	**C1**	-	0	**BLS.2**	-	0
10.7 (P)	+	1.7	**C2**	-	0	**BLS.3**	-	0
4Su.10 (P)	++	1.6	**C3**	+	0.9	**BLS.4**	-	0
W3.10 (c)	+	2	**C4**	+	1.4	**BLS.5**	-	0
10.10 (P)	+	1.1	**C5**	-	0	**BLS.6**	-	0
12.2 (P)	-	0	**C6**	-	0	**BLS.7**	-	0
4.7 (P)	+	1.5	**C7**	+	0.6	**BLS.8**	-	0
7.13 (P)	+	0.5	**C8**	+	1.1	**BLS.9**	-	0
C1.15 (C)	-	0	**C9**	+	0.9	**BLS.10**	-	0
8.9 (P)	+	1.8	**C10**	++	1.5	**BLS.11**	-	0
11.8 (L)	+	1.5	**N1**	-	0	**BLS.12**	-	0
3.9 (P)	-	0	**N2**	-	0	**BLS.13**	-	0
4Su.3 (P)	+	0.5	**N3**	-	0	**M2.1**	-	0
W3.24 (C)	+	1.1	**N4**	-	0	**M2.2**	-	0
7.11 (P)	+	1.2	**N5**	-	0	**M2.3**	-	0
10.3 (P)	+	1	**N6**	-	0	**M2.4**	-	0
12.6 (P)	+	0.4	**N7**	-	0	**M2.5**	-	0
1Su.10 (P)	-	0	**BLR.1**	-	0	**M2.6**	-	0
4.5 (P)	+	2.3	**BLR.2**	-	0	**M2.7**	-	0
1.4 (P)	+	1.2	**BLR.3**	+	1.1	**M2.8**	-	0
7.11 (P)	+	1.2	**BLR.4**	+	1	**M2.9**	-	0
4.4 (P)	+	2.2	**BLR.5**	-	0	**M2.10**	-	0
4SU.2 (P)	+	0.5	**BLR.6**	-	0	**M2.11**	-	0
4Su.11 (P)	+	0.6						

Upon quantitative screening 16 fungal and 18 bacterial strains were found to produce high amount of chitinase enzyme. Out of which MG23.1, MG23.3 and BH fungal isolates were producing maximum amount of chitinase while E1and SN13 were selected bacterial strains for chitinase production.

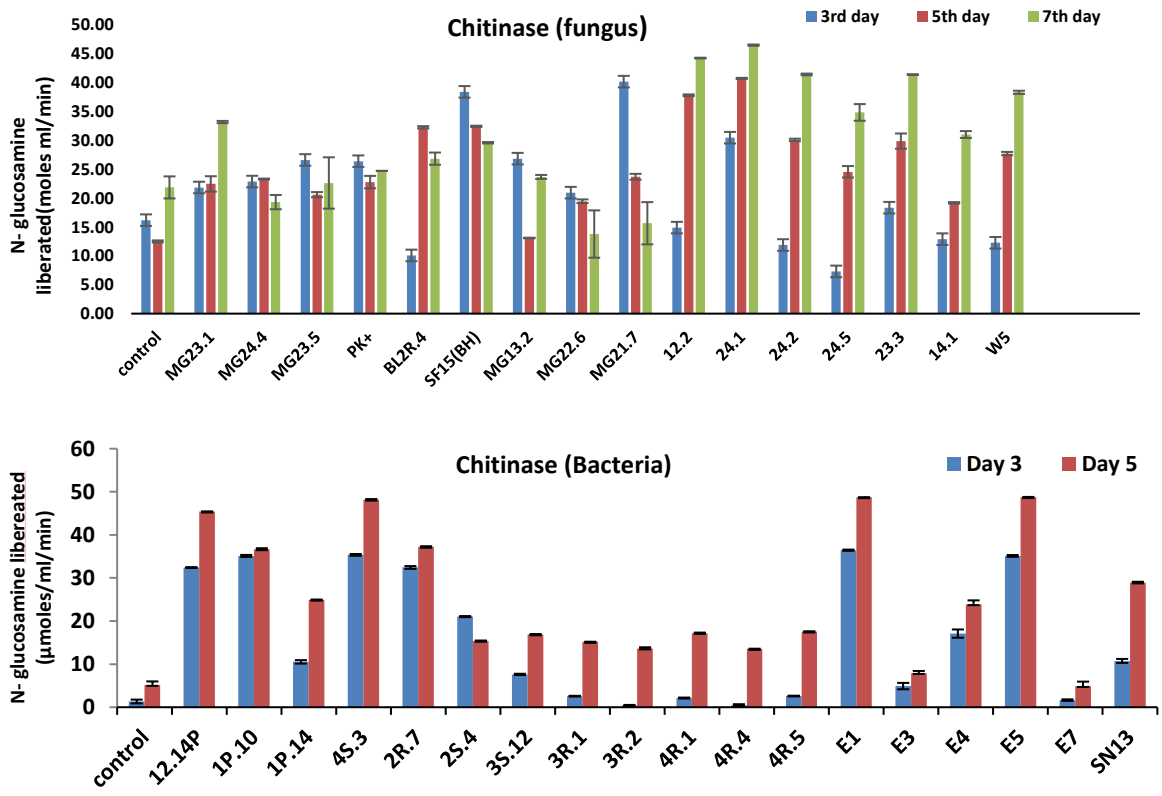

Figure 8: Quantitative estimation of Fungal and bacterial strains for chitinolytic activity

4.3 Fermentation Process for Chitinase Production

Two types of fermentation processes had been used for Chitinase production *viz.* Sub-merged Fermentation (SmF) and Solid State Fermentation (SSF) technology. Among SMF and SSF technology, solid state fermentation (SSF) show better response for the Chitinase enzyme production, according to the result i prefer SSF technology above SmF technology. The both fungal strains (23.1 & BH) show higher chitinase activity in corn cob of about 41.8 and 38.5 µmoles of N-glucosamine produced/ml/min after 20 days of incubation.

The logical reasoning behind this selection is that, SSF microbial cultures are closer to their natural habitat and probably hence their activity is increased (R.P. Tengerdy, 2003) also in SSF, solid material is non-soluble that acts both as physical support and source of nutrients (Luciana P.S Vandenberghe et. al. 2000)

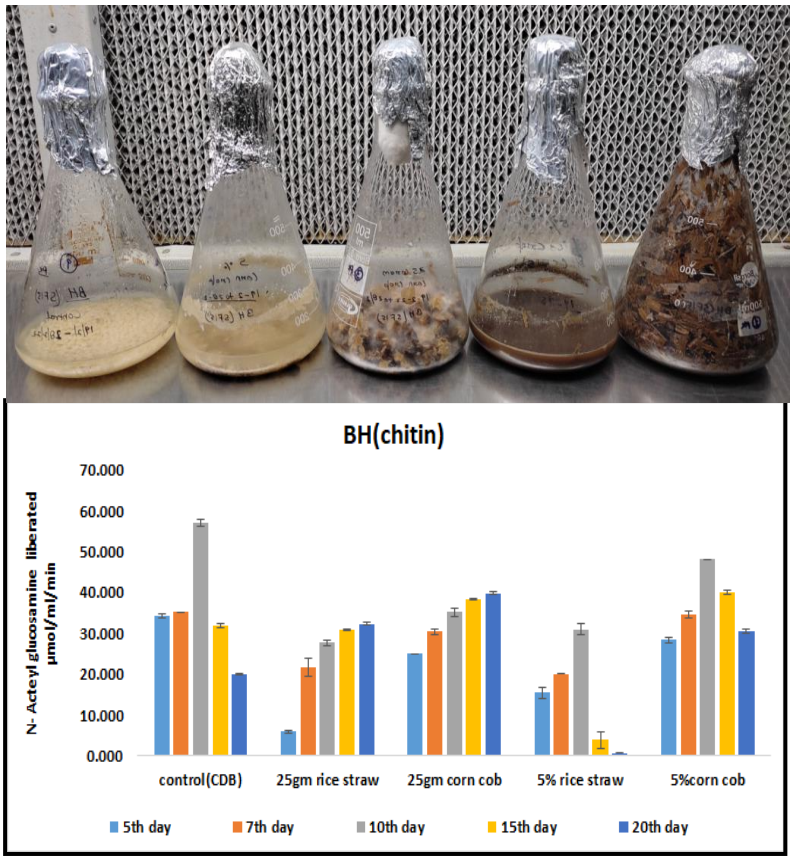

Figure 9: SmF and SSF technology using corn cob and rice straw with BH fungal strain

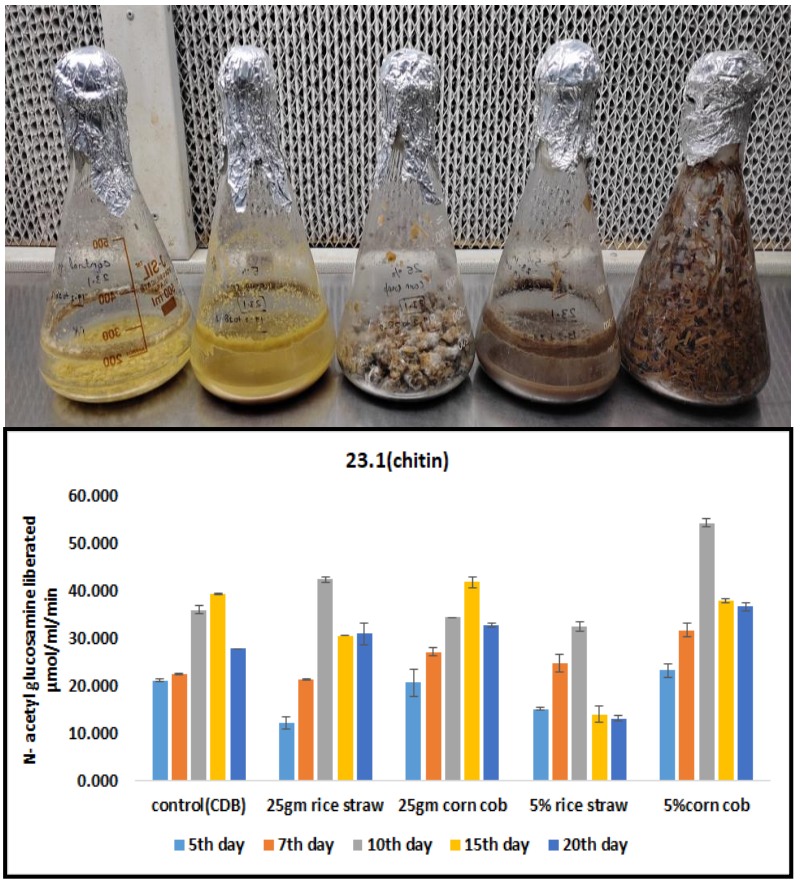

Figure 10: SmF and SSF technology using corn cob and rice straw with MG23.1 fungal strain

4.4 *In-vitro* compatibility test for efficient consortia:

In-vitro compatibility test was performed in order to obtain an efficient fungal-bacterial consortium that can elevate the chitinase production compared to single strain. Four fungal *viz.* MG23.1, MG23.3, MG12.2 and BH and two bacterial strains *viz.* E1 and SN13 were selected for compatibility test.

MG23.1 was found compatible with both SN13 and E1 while MG23.3 strain was compatible with E1 and SN13 both. BH fungal strain had shown compatibility with E1 and was inhibited by SN13 strain.

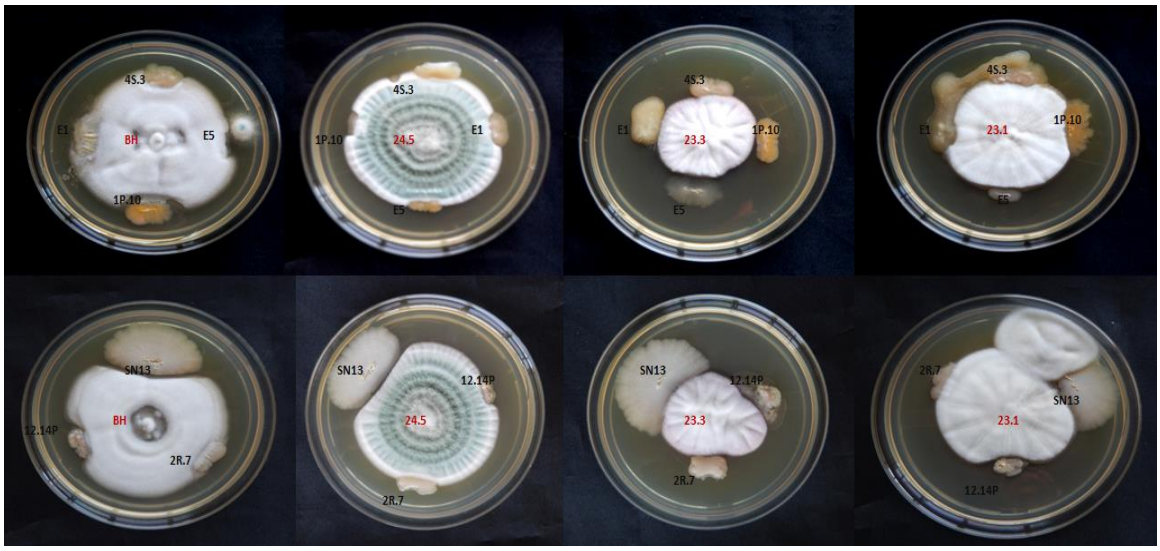

Figure 11: Compatibility test among fungal and bacterial strains

4.5 Up-scaling process of production of Chitinase enzyme

Up- scaling for chitinase production was accomplished using increased amount of flask i.e. 100gm of corn cob, rice straw and bagasse in 5lt flask maintaining moisture accordingly.

4.5.1 *Corn cob*

Figure 12: Up-scaling for chitinase production using corn cob

Four consortia had been used for enzyme production. The enzyme was extracted using 20 ml Sodium phosphate citrate buffer with 5 gm substrate. The same extract was used for chitinase estimation. Consortium BH+ E1 was producing highest amount of chitinase enzyme followed by 12.2+ SN13 consortiums. Also to observe the ability of degradation by consortium and additional cellulase and laccase production assay was performed for cellulose and lignin respectively. BH+ E1 produced maximum cellulose at 3rd day of incubation but the enzyme production decreased from 5th day while 12.2+SN13 is producing less enzyme but the production was maintained till 10th day. Laccase enzyme production was increasing in 23.3+ SN13 consortium from 3rd day till 10th day.

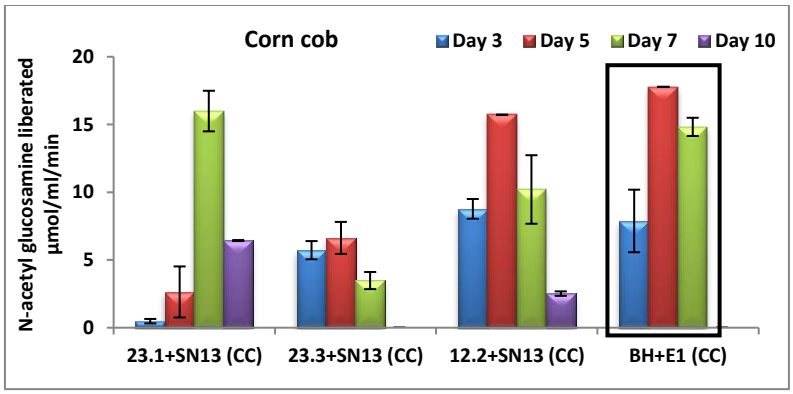

Figure 13: Graph showing chitinase production using four consortia with corn cob as a substrate

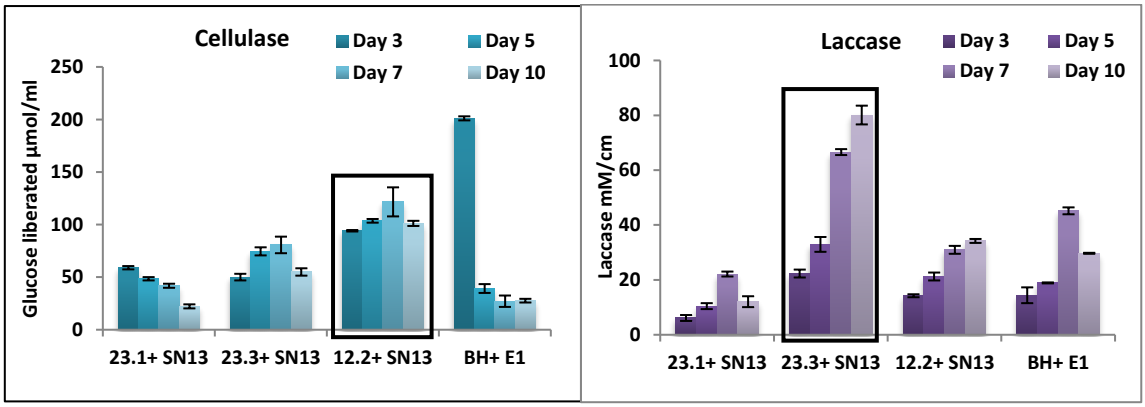

Figure 14: Graph showing cellulase and laccase production using four consortia with corn cob as a substrate

4.5.2 ***Rice straw***

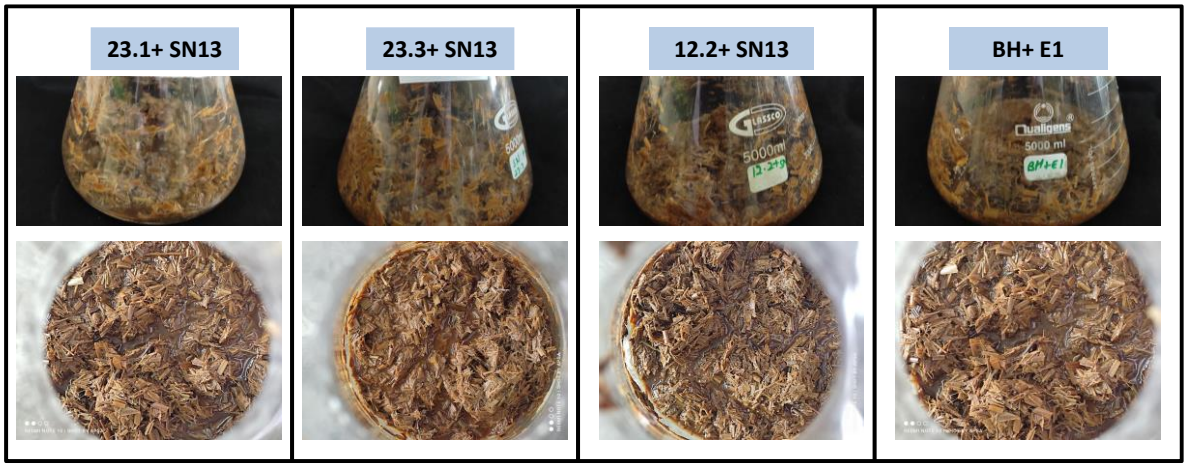

Figure 15: Up-scaling for chitinase production using Rice straw

Four consortia had been used for enzyme production. The enzyme was extracted using 20 ml Sodium phosphate citrate buffer with 5 gm substrate. The same extract was used for chitinase estimation. Consortium 23.1+ SN13 was producing highest amount of chitinase enzyme with 57.5 µmol/ml followed by 12.2+ SN13 consortiums. The same degradative enzyme assays had been performed for rice straw extract and it was observed that consortium 23.1+SN13 was producing maximum amount of laccase enzyme on 10th Day of inoculation while cellulase production was also recorded maximum by same consortium on 7th day.

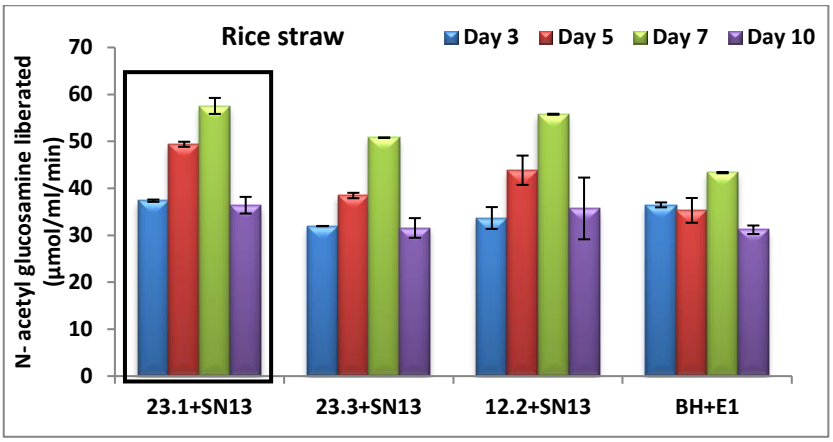

Figure 16: Graph showing chitinase production using four consortia with Rice straw as a substrate

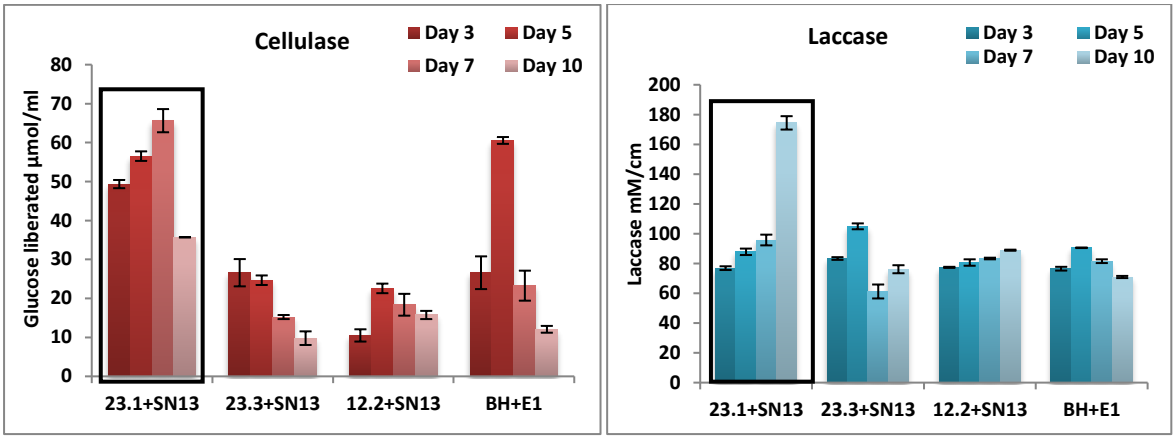

Figure 17: Graph showing cellulase and laccase production using four consortia with Rice straw as a substrate

4.5.3 *Sugarcane Bagasse*

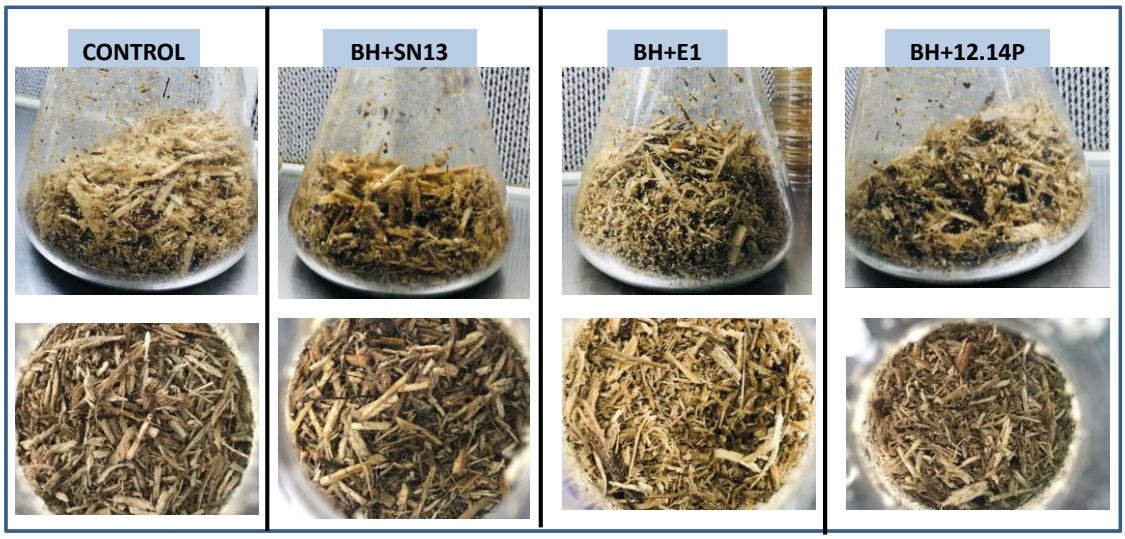

Figure 18: Up-scaling for chitinase production using bagasse

Fungal strain BH had been inoculated with three different bacterial strains 12.14P, SN13 and E1 with 100 gm bagasse as a source of carbon. The chitinase production was estimated at 3rd, 5th, 7th and 10th day. It was observed that consortium BH+ 12.14P was producing and maintaining constant chitinase production from 1st day to 10th day followed by consortium BH+ SN13. The growth of consortia is also marked by production of degradative enzymes- 1) cellulase for cellulose and hemicellulose 2) Laccase for lignin. Laccase production had been seen at peak on day 10 in presence of BH+ SN13 while a slight less production in BH+ 12.14P consortium while cellulase production was found maximum in case of BH+ E1 at 10th day of incubation.

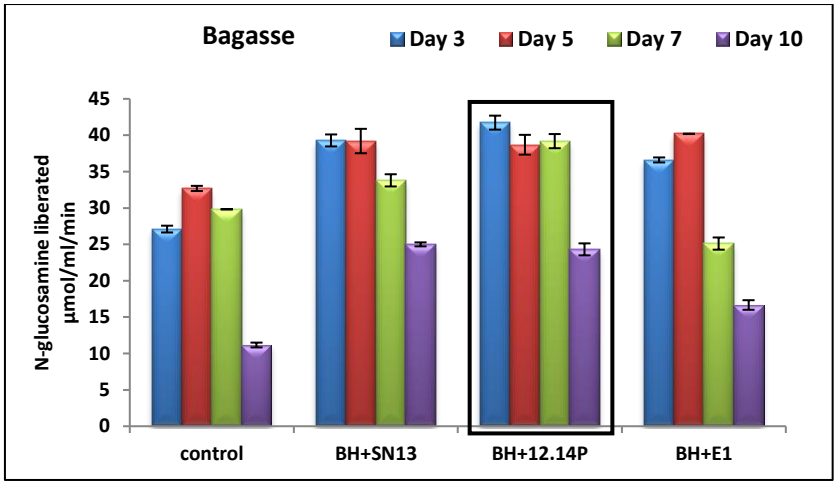

Figure 19: Graph showing chitinase production using four consortia with bagasse as a substrate

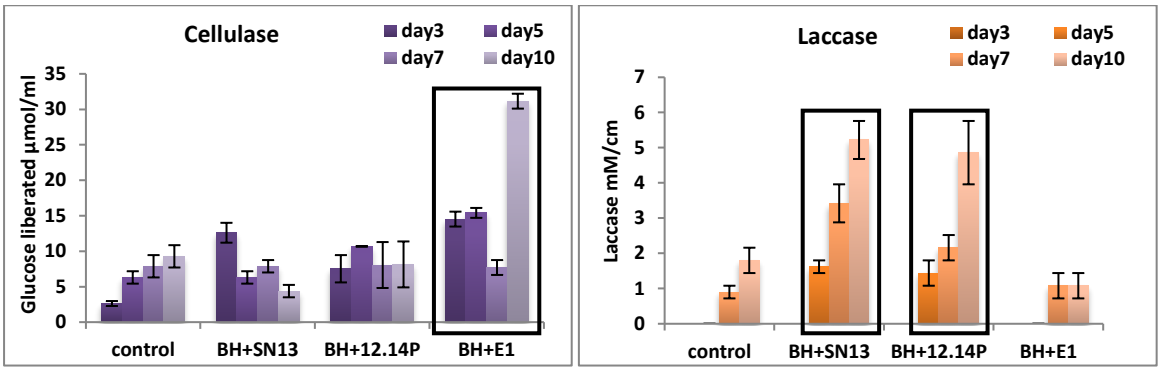

Figure 20: Graph showing cellulase and laccase production using four consortia with bagasse as a substrate

4.6 Extraction and evaluation of crude enzyme

The extracted crude enzymes obtained by ammonium Sulphate precipitation of corn cob extract and sodium acetate buffer were further tested for antifungal activity. Crude enzyme produced by consortium BH+ E1 showed efficient antifungal activity at 100 µl concentration while 23.3+ SN13 and 23.1+ SN13 showed maximum effectiveness at 200 µl. A control plate runs along with every treatment which showed unhindered growth of *Rhizoctonia solani*. The same assay for antifungal activity was performed for rice straw and bagasse. The crude enzyme from rice straw has shown promising results as compared to enzyme produced via bagasse.

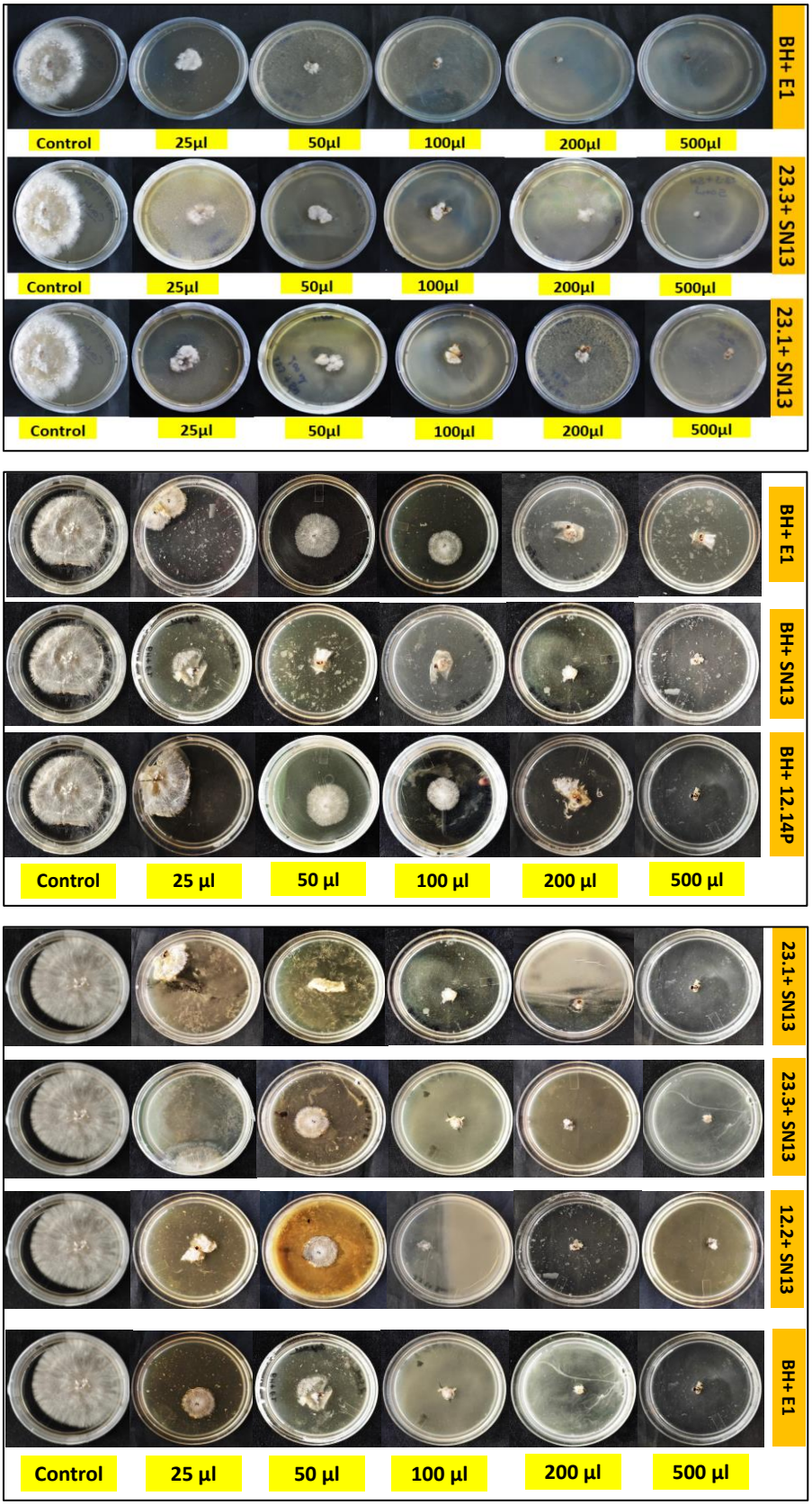

Figure 21: Evaluation of antifungal activity by crude enzyme from corn cob, bagasse and rice straw

4.7 Molecular characterization of microbial strains

The extracted DNA of microbial strains had been amplified using ITS and 16s primers specific for fungus and bacteria respectively. The amplified DNA was being viewed on UV trans-illuminator and the gel image is depicted in fig. 22. Further DNA samples were out sourced for sequencing (in progress).

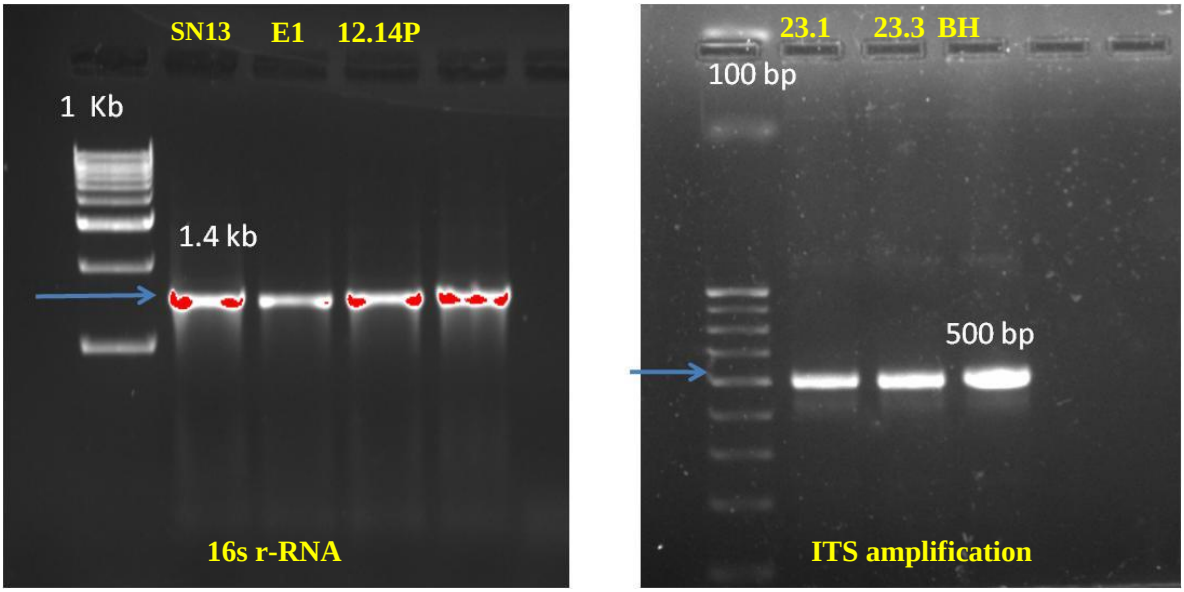

Figure 22: Gel image showing the amplification of ITS and 16s sequences

CONCLUSION

- Out of total fungal and bacterial strains four fungal 23.1, 23.3, 12.2 and BH; three bacterial 12.14P, E1 and SN13 were selected as best Chitinase producing strains.
- Among SSF and SMF, SSF technology was seen to produce maximum amount of enzyme.
- All three Agri wastes can be used to produce Chitinase in large amount, but rice straw was seen to produce a little more enzyme than the other two.

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